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GRANT NUMBER: DAMD17-94-J-4265

TITLE: Active Follow-Up of Participants of a Population-Based Specimen Bank (Plasma, DNA, RBC)

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REPORT DATE: October 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Annual (1 Oct 95 - 30 Sep 96)

4. TITLE AND SUBTITLE
Active Follow-Up of Participants of a Population-Based Specimen Bank (Plasma, DNA, RBC)

5. FUNDING NUMBERS
DAMD17-94-J-4265

6. AUTHOR(S)
Kathy J. Helzlsouer, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Johns Hopkins University
Baltimore, MD 21205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The objectives of this proposal are to expand the resources of our population-based specimen bank (CLUE II) by collecting additional information on family history and breast cancer risk factors and to continue plasma stability studies.

From May through November 1989, 32,320 blood specimens were collected from residents of Washington County, Maryland and the surrounding tri-state area. Participants donated 20 ml of blood, gave a brief medical history, completed a food frequency questionnaire and provided a nail sample. An active follow-up of the cohort will be instituted to collect and update information on breast cancer risk factors, including an extensive family history. Stability studies of Micronutrients and hormones in years 5 and 9 from the time of initial blood collection will be conducted.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	40
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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N/A ____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A ____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A ____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Kathy J. Nelson 10/21/96

PI - Signature Date

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INTRODUCTION

Objective:

The purpose of this proposal was to expand the resources of our existing population-based specimen bank by updating baseline information and obtaining information on breast cancer risk factors so that gene-environment interactions leading to breast cancer may be investigated.

Background of Previous Work:

The CLUE Specimen Banks

From August through November, 1974, a total of 25,620 serum specimens were collected in Washington County, MD for a research serum bank (CLUE I). An additional 182 specimens were obtained in the summer of 1975, for a total of 25,802. Linkage of the records from this program to those of a private census in the summer of 1975 indicated that almost a third of the adult population of the county had participated. Participation was best in the age group 35 to 65 years, and was slightly better among females, the better-educated, and nonsmokers.

A second program was conducted from May through November, 1989 collecting 32,320 blood specimens (CLUE II). Of these specimens, 8118 were collected from individuals who had also participated in CLUE I. CLUE II participants donated 20 ml of blood, gave a brief medical history, completed a food frequency questionnaire and returned a toenail clipping for trace metal studies. The blood specimens have been stored at -70° C. Somewhat greater participation was obtained among older persons, possibly because a free cholesterol test was offered as an incentive to participate. Quality checks showed good agreement between cholesterol determinations by the local medical laboratory and the CDC-approved laboratory at Johns Hopkins Hospital.

In addition to storing two 5 ml aliquots of plasma, another 0.7 ml aliquot was preserved with 0.7 ml of 10 % metaphosphoric acid to allow subsequent ascorbic acid assays. The buffy coat (providing DNA) and an aliquot of red blood cells were also stored. The two aliquots of plasma from each person are stored in separate freezers.

Clue I has been extensively used to examine the potential protective effect of specific micronutrients against the development of cancer including breast cancer (1). The role of endogenous hormones in the development of breast cancer has also

been examined using the resources of this serum bank (2). With the maturation of the cohort from CLUE II, the availability of DNA in addition to plasma, and the technological advances of molecular biology we will be able to investigate etiologic, protective and susceptibility factors leading to the development of breast cancer. In order to use the specimen bank to its fullest potential we require additional data on participants in the CLUE II cohort such as extended family history and other risk factors. Breast cancer risk factors such as family history of disease, age at pregnancy and parity change over time requiring institution of active follow-up of the cohort. These risk factors should be taken into account in investigations of serologic precursors or susceptibility factors associated with breast cancer. The ability to identify and investigate families with multiple members affected by breast cancer is a valuable resource for studying the role and contribution of inherited susceptibility factors to the development of breast cancer. Since these inherited factors may be passed through the mother or father it is essential to obtain information on the family history of cancer of male and female participants in the cohort.

Stability studies of micronutrients and hormones have been completed up to 42 months. We proposed to continue these studies up to 15 years. The resources of serum and specimen banks become more valuable with time as the number of cancer cases developing among member increases with aging of the cohort. It is essential to assess the stability over many years of factors being studied in order to optimally utilize these valuable resources. A literature review of the effects of long-term freezer storage on concentrations of retinol, beta-carotene, and alpha-tocopherol in repeated assays of the same serum or plasma pools shows that most of the studies are deficient because of very small numbers of observations, imprecise descriptions of procedures, and/or short periods of storage (3). The literature is even more scanty with respect to long term storage effects on concentrations of other carotenoids, ascorbic acid, and hormones. It is essential to have this information to satisfy study sections and reviewers of manuscripts. In our experience over the past 20 years, such queries have been almost universal.

Cancer Register:

A cancer register for Washington County has been maintained since 1958, with records dating back to 1948. Its primary source is discharge records from the Washington County Hospital, the only general hospital in the county. Because of its well-equipped and staffed Oncology Service, the hospital tends to draw patients from surrounding counties rather than to lose them to other institutions. Cases are also ascertained from death certificates of Washington County residents which are under the

custody of our unit acting as a branch of the Health Department. Comparisons of observed cases in the populations that donated blood for the serum bank with the number expected on the basis of race-sex age specific rates from the SEER registries suggest that reporting is essentially complete for this subpopulation. The only major deficit is for stomach cancer; the only major excess is for prostate cancer. Records of reported county cancer cases are computer-linked to the lists of serum bank donors. Matching on
variables such as age, date of blood donation, day of menstrual cycle are readily accomplished. Age matching is often possible within a few days or weeks.

Purpose of Present Work

Further progress in understanding the etiology of breast cancer and in developing new methods for early detection and prevention requires investigations to consider both genetic and environmental influences and their interaction in the development of breast cancer. The purpose of this proposal is to expand the resources of our existing population-based specimen bank by updating baseline information and obtaining information on breast cancer risk factors, particularly family history of cancer. Gene-environment interactions may then be explored to the fullest potential. The availability of family history information will permit the targeting of high risk individuals, based on familial factors, for investigations of possible inherited susceptibility factors.

The second major objective of this proposal is to continue to obtain fundamental information on changes in the concentration of various analytes in plasma and blood cells associated with long-term storage at -70° C. The plasma analytes are retinol, ascorbic acid, the major carotenoids found in human serum, alpha- and gamma-tocopherol,sex-hormone-binding globin, and selected steroid hormones. The hypothesis to be tested in each case is the null hypothesis that there will be no change in concentration with storage time. The resources of banks become more valuable with time as cohorts mature providing more cases of cancer for investigation, and as new hypothesis and techniques become available for evaluation. Storage studies will provide basic information on the stability of markers of exposure, susceptibility and protection from breast cancer that is almost nonexistent. This information is essential for planning and interpreting studies using stored plasma.

Technical Objectives (Specific aims)

To enhance the resources of the existing population-based specimen bank we propose to:

1. Expand information collected at CLUE II in 1989. For example, total years of smoking and medication use, especially exogenous hormones.

2. Update information collected at CLUE II. For example, total years of smoking and medication use, especially exogenous hormones.

3. Obtain additional information relevant to breast cancer. For example, detailed cancer family history, number and timing of pregnancies, preventive health behavior (breast cancer screening), history of breast biopsies and occupational exposures.

4. Expand the cancer registry population base including CLUE II participants who reside outside of Washington County.

To facilitate the appropriate use of the resources and to enhance interpretation of studies performed on the cohort we propose to:

5. Continue the study of the effects of long-term freezer storage of plasma at -70° C on its content of antioxidant nutrients, such as retinol (including retinoic acid and retinol palmitate), ascorbic acid, carotenoids, and tocopherols, as well as its content of hormones and lipoproteins.

Methods

Eligible Population

CLUE II participants living within a 30 mile radius of downtown Hagerstown were included in the active follow-up cohort. This geographic boundary includes all of Washington County and parts of surrounding counties, extending into Pennsylvania and West Virginia. This boundary includes 30,100 of the 32,320 CLUE II participants. This boundary was chosen to form a homogenous cohort for long-term follow-up. Of the CLUE II samples excluded from the cohort, 2,200 will be used for our storage studies and cross-sectional investigations that can be conducted using data collected at baseline.

Follow-up Procedure

CLUE II participants located in the geographic boundary of the long-term follow-up cohort will be mailed a questionnaire approximately every 2 years. It will take a considerable amount of clerical work to update addresses from the time of CLUE II participation since all rural route box numbers have been changed to street (road) addresses.

Non-respondents will be sent a second questionnaire. Telephone follow-up will be carried out to clarify questionnaires completed by respondents. The resources available to search for current addresses of participant's include the Polk's City Directory for Hagerstown which covers Washington County and surrounding communities, telephone directories, and the Hill-Donnelly Cross Reference Directory.

As part of this study, we propose to collect additional data on CLUE II participants. Specifically, data will be collected from women on known risk factors for breast cancer. Examples of risk factors include: family history of cancer in first and second degree relatives, history of breast biopsy, type of benign breast disease, age at menarche, first birth, menopause, parity, months of lactation, use of exogenous hormones, height and weight and screening history. Updated risk factor information will be obtained at approximately 2 year intervals. Updated food frequency information will be obtained at the second mailing. Because inherited susceptibility factors may be inherited through the mother or the father and because of recent evidence of common inherited and environmental factors for breast and prostate cancer we will include male and female participants in the active follow-up cohort.

Information will be obtained from participants using a self-administered mailed questionnaire. A second questionnaire will be mailed to non-respondents. Deceased CLUE II participants will be identified by linking the population to the mortality data base and by using obituaries from the local newspaper. The Training Center for Public Health Research is a repository for county death certificates.

Machine readable forms will be used for all questions except family history. Questions related to family history are not practical in a machine readable format because name, age at diagnosis, and all causes of death must be recorded.

Methods for Studies of Stability

The campaign for participation in CLUE II was conducted from May to November 1989. Blood was drawn into a 20 ml vacutainer containing heparin and was refrigerated at 4 C until it was delivered to the CLUE II laboratory within a few hours after being drawn. After centrifugation, 0.7 ml of plasma was divided into two equal aliquots, the buffy coat was removed, as were 2 ml of red blood cells. Each of these specimens were placed in cryotubes and promptly frozen at -70° C.

For the stability studies of micronutrients and hormones, pools of plasma were created from the plasma of persons who lived outside of the study area and who had donated blood near the end of the campaign. For the micronutrients, 40 pools were created, each containing the plasma from four individuals. The 40 micronutrient pools and the 16 hormone pools were each composed of equal numbers of pools from young and old men, and young and old women. A large reference pool was also created so that four aliquots could be added to the micronutrient specimens and tow to the hormone specimens.

Assays for micronutrients and hormones have been done at baseline, 12, 22, and 42 months after the median time of blood drawing. We propose to do three more rounds of assays. The assays will be done in the same laboratories at each round, using the same methods insofar as this is practical. Micronutrient assays have been done in the laboratory of Dr. Edward Norkus at Our Lady of Mercy Medical Center in New York City using high performance liquid chromatography. The micronutrients assayed included retinol, alpha-and gamma-tocopherol, the carotenoids, and ascorbic acid. The carotenoids included total carotenoids, alpha- and beta-carotenes, cryptoxanthin, lutein, and lycopene (4). Ascorbic acid was assayed by Dr. Norkus on plasma preserved with meta-phosphoric acid using high performance liquid chromatography (5). The hormone assays have been done in the laboratory of Dr. Christopher Longcope, University of Massachusetts. Assays include estrone, estradiol, testosterone, and progesterone (6). Inter-and intra-assay coefficients of variation for these assays are approximately 10% and 7% respectively. Sex hormone binding globulin capacity will be measured using the filter disc method of Mickelson and Petra (7). Inter-and intra-assay coefficients of variation are reported to be 10.9% and 8.0% respectively (8).

The basic analysis of the data was to calculate regression lines for each pool and to average the slopes of each age-sex group, and when appropriate, for the total group. This approach assumes that an equal amount of analyte is lost in each unit of time. Other assumptions above the rate of change can be made, but have not yet been deemed necessary.

BODY: PROGRESS REPORT YEAR 2

Task 1: Identification of Eligible Population

CLUE II participants living within a 30 mile radius of downtown Hagerstown were included in the active follow-up cohort. This geographic boundary includes all of Washington County and parts of surrounding counties, extending into Pennsylvania and West Virginia. This boundary includes 30,110 of the 32,319 CLUE II participants. This boundary was chosen to form a homogeneous cohort for the long-term follow-up. Persons under the age of 13 at the time of participation were excluded from the follow-up cohort because of issues regarding informed consent. The Clue II participants who met the age requirement, but who reside outside of the 30 mile radius were excluded from the follow-up cohort (2,220). The master data file was coded according to geographic location into three categories: 1) Washington County residents, 2) persons residing outside of Washington County but within 30 miles of downtown Hagerstown, and 3) persons residing more than 30 miles from Hagerstown.

Deceased CLUE II participants were identified by linking the CLUE II population to death certification data, and by using obituaries from the local newspaper. Approximately 1,186 persons or 4 percent of the eligible population were determined to be deceased. Another 150 were reported to be deceased by a family member. We have not sent a questionnaire to next-of-kin for this group.

Task 2: Tracing of Participants

The process of actively updating participant addresses started in August of 1995. Staff were assigned work by alpha-numeric CLUE II number. Computer print outs were prepared and the address written on the print out if found. The address was then entered into the appropriate computer data base. In order to simplify work procedures, we divided the cohort into three groups; 1) Washington County residents, 2) persons residing outside of Washington County, but within the 30 mile radius, and 3) the group of
participants who were between the ages of 13 and 18 years of age at the time of participation. This group was separated so that a letter could be sent to a parent or guardian requesting a current address. We obtained a 37 percent response rate from parents or guardians who provided us with a current address.

The resources that were used to search for current addresses of participant's were the Polk City Directory for Hagerstown, telephone books, the Hill-Donnelly Cross Reference Directory, and a computer printout obtained from the Washington County Planning Commission of tax listings for homeowners in the county. An

attempt was made to identify name changes for females by searching wedding announcements which are published in the local newspaper. We were able to trace 80 percent of the eligible cohort using these resources.

Task 3: Questionnaire Development

The questionnaire was developed during the months of January and February 1996. A draft of the questionnaire was pilot tested by mailing it to the approximately 2,170 individuals who lived outside the 30 mile radius of Hagerstown.

Since the length of the questionnaire was an important concern, we evaluated the effect of questionnaire length on response rate during the pilot testing of the questionnaire. Two thirds of the pilot group (N=1504) were sent a long questionnaire consisting of 17 pages of questions. The remaining one third was sent a shorter version of the questionnaire which included 4 pages of questions on demographics, medical history, and family history of cancer. Participants were assigned to a group based on the terminal digit of their CLUE II number.

Questionnaires were mailed over a five day period and those with address corrections were promptly remailed to the new address. Of the questionnaires returned as non-deliverable (N=653), 30 per cent were excluded from the analysis. The cutoff period for recording responses was 21 days after the initial mailing. The response rate for the long version of the questionnaire was 32 per cent compared to 35 per cent for the shorter version of the form. We concluded that the shorter version of the questionnaire did not significantly increase response rate, so we decided that it was not necessary to shorten the questionnaire.

During the 21 day time period allowed for pilot test responses, the overall response rate was 33 per cent. Data entry of the pilot questionnaires was started as soon as they were returned. This process allowed us to identify problems with the wording of questions as well as potential coding problems. Once data entry was completed and problems recorded, modifications were made to the questionnaire and it was sent to the printer for final graphic alterations and printing. The graphic alterations and printing of the questionnaire took longer than anticipated largely because of problems with the printer of the machine-readable questionnaire.

Task 4: Mailing of the Newsletter and Questionnaire

The newsletter was mailed in April, 1996, two months prior to mailing the questionnaire. Mailing the newsletters with sufficient time to make address corrections before mailing the questionnaire served two purposes: 1) it allowed time to prepare an updated disk from which name and address could be laser-printed on the form at the time of questionnaire printing, and 2) address corrections were requested for participants, which were useful in correcting the address data base and prevented the expense of mailing a questionnaire to an incorrect address. It took a couple of weeks to update the address data base after the newsletter mailing.

The local newspaper, television and radio stations proved to be very cooperative in promoting the follow-up study. The newspaper published a front page article, and the local television station covered the story on the evening news on two occasions. Additionally we participated in two radio interviews.

Study of Effects of Material Incentives on Response Rate

Prior to the questionnaire mailing, a pilot study was conducted to test the effect of including a newspaper article and\or a special pencil on the response rate to the questionnaire. The study group was divided into five geographic regions by zip code and stratified by study number within each zip code. Within each zip code the first 100 persons were selected to receive one of four interventions, (a pencil, a copy of a newspaper article which described the study and its importance, both the article and pencil, and no intervention. Assignment to an intervention group was based on the terminal digit of the house number.

There were a total of 2,000 persons included in the pilot study. Four weeks after the mailing we found that there were no significant differences in response rate among the four groups. Based on these findings we decided to spare the expense of including an incentive in an attempt to boost the response rate. A summary of the results are as follows:

Table 1

Effect of Incentive on Response Rate After Four Weeks

	Responded	Total*	%
No intervention	241	474	51
Pencil	245	472	52
Newspaper	253	487	52
Pencil and newspaper	257	488	53

* Questionnaires returned as non-deliverable were not included.

Mailing of Questionnaires

To simplify sorting, questionnaires were mailed by zip code beginning on July 12, 1996. Assembly and mailing occurred over a five week period at the rate of approximately 5,000 per week. The final group was mailed during the week of August 19, 1996. As of mid October we have received approximately 14,000 completed questionnaires. The response rate is 59 percent. In an attempt to obtain addresses for persons for which we were unable to locate a current mailing address, we ran an article in the newspaper requesting that persons who participated in CLUE II but did not receive a questionnaire contact us. We received a number of phone calls and promptly mailed a questionnaire.

Task 5: Follow-up of Non-Respondents

Non-Respondents from the original pilot study group were used to test the effectiveness of a postcard reminder versus a second questionnaire and a letter. This group was composed of 873 persons. They were allocated to a study group based on the terminal digit of their study number.

Second questionnaires were sent to 430 persons. Post card reminders were sent to 443 persons. After four weeks, 27 percent of those who were mailed the second questionnaire responded, whereas 15 percent of those who were sent the post card responded. Our research suggests that adding a questionnaire in a second mailing has a significant effect on response rate.

During the first week of October we sent a second questionnaire to approximately 11,000 persons who had not responded.

Task 6: Data Management

Editing of the returned questionnaires has started. Telephone calls are being made to respondents to clarify responses.
Data entry of those sections of the questionnaire which cannot be scanned is well underway. The last month has been spent setting up the scanner, loading the software and writing the form description. Procedures for data management are currently being developed.

Task 7: Study of effects of stability of plasma components when stored at -70° C

Participants in CLUE II donated 20 ml of blood in 1989. This was collected in a 20 ml vacutainer containing heparin and was stored at 40° C until it was delivered to the laboratory within a few hours after being drawn. After centrifugation, 0.7 ml of plasma was added to 0.7 ml of 10% metaphosphoric acid for ascorbic acid assays. The remaining plasma was divided into two equal aliquots, the buffy coat was removed, and 2 ml of red blood cells was saved. Each of these specimens was placed in 5 ml cryotubes and promptly frozen at -70° C.

For the stability studies of micronutrients, hormones, and apolipoproteins A and B, pools of plasma were created from specimens donated by persons who lived far outside the study area and who had participated near the end of the campaign. For ascorbic acid assays, 40 pools were created, each containing the plasma from four individuals. For the other assays, 16 pools were created, again each containing the plasma from four persons. For all of the analytes, one quarter of the pools was composed of plasma from each of the four sex-age groups (males born 1910-1939, males born 1949-1969; females born 1910-1939; and females born 1940-1969). A large quality control pool was also created so that four quality control aliquots could be included in the ascorbic acid assays, and two in the other assays.

Ascorbic acid assays were done after 12, 24, and 42 months of freezer storage. Other micronutrients were assayed after 15.5, 27.5 and 51.5 months. These results have been reported (9). Briefly, they show that there was no indication of any meaningful losses of ascorbic acid, retinol, alpha-carotene, beta carotene, cryptoxanthin, lutein, lycopene, alpha-tocopherol, or gamma-tocopherol during the approximately 4 years of storage. A fourth series of assays has just been completed.

The situation with respect to hormones is unclear, to put it mildly. Estrone, estradiol, progesterone, testosterone, and sex hormone binding globulin were assayed after 6, 12, 31, and 78

months of storage. The mean values for all 16 aliquots are shown in table 2 along with minimum and maximum values at each time, and the coefficients of variation for the two quality control specimens. Most of these coefficients are within reasonable limits, although six of the 20 exceed 15%. Most troubling are the marked increases in concentration with long-term storage for estrone and estradiol, and the sharp drop at 78 months in the concentration of progesterone. There has been no evidence of loss of water during storage.

With respect to apolipoproteins A and B, the quality control assays showed very little variability at any of the individual time periods. However, there was a marked increase in concentration of both analytes from 11 months to 54 months of storage. Values at 24 months are difficult to interpret because a different method was used, and the correction equation proposed by the laboratory does not agree with the correction equation derived by the results in this study when the 24 month specimens were assayed by both the old and new methods.

We plan to prepare a short note for publication regarding the stability (or lack of it) for the micronutrients as soon as analysis of the most recent assays are completed. With respect to hormones, we plan to re-assay aliquots of serum or plasma previously assayed by the same laboratory that has done the storage effects study. If the finding of marked changes is confirmed, this will have major consequences for studies of hormones in stored plasma or serum. Whether or not to do 6-year assays of apolipoproteins will depend on whether or not the laboratory can still do the original method reliably.

Table 2

Mean values, standard deviations, minimum and maximum values for selected hormones in 16 different plasma pools assayed after 6, 12, 31, and 78 months of storage at -70° C, Clue II, Washington County, MD

Months of storage	Summary values for 16 pools				
	Mean	S.D.	Minimum	Maximum	Coeff. of variation*
			ESTRONE (pg/ml)		
6	33.0	13.0	16	54	8.1
12	24.1	9.0	12	42	5.7
31	45.0	21.2	16	93	19.4
78	73.5	24.3	41	121	29.4
			ESTRADIOL (pg/ml)		
6	28.0	15.2	11	64	10.9
12	19.9	9.6	7	44	12.5
31	56.4	28.6	30	128	3.9
78	55.4	32.1	28	134	52.9
			PROGESTERONE (ng/ml)**		
6	4.06	3.43	1.60	8.92	8.7
12	4.80	2.61	1.05	6.74	63.6
31	4.24	3.74	1.06	9.26	6.2
78	1.24	0.51	0.68	1.84	29.4
			TESTOSTERONE (ng/ml)		
6	2.25	2.41	0.03	6.66	4.1
12	1.98	1.92	0.16	5.72	10.4
31	2.91	2.82	0.18	7.29	2.3
78	2.48	2.51	0.16	6.71	4.2
			SEX HORMONE BINDING GLOBULIN (nmol/L)		
6	56.4	22.9	28.5	99.8	7.9
12	55.7	21.7	28.9	95.5	16.5
31	54.5	19.2	29.4	96.0	4.1
78	49.3	17.0	29.7	87.3	1.0

* In %, based on 2 aliquots of quality control plasma from same pool.

** Based on the four pools from young females (ages 20-49 years). Other age-sex groups were excluded because values were extremely low and often below the level of detection.

Table 3

Mean values, standard deviations, minimum and maximum values for apolipoproteins A and B in 16 different plasma pools assayed after 11, 24, and 54 months of storage at -70° C, CLUE II, Washington County, MD

Months of storage	Summary values for 16 pools				
	Mean	S.D.	Minimum	Maximum	Coeff. of variation*
			APOLIPOPROTEIN A		
11	140.7	16.1	116	169	1.0
24	152.3	22.4	121	191	6.3
54	194.7	54.8	122	327	5.6
			APOLIPOPROTEIN B		
11	91.8	77.2	68	113	0.0
24	108.8	12.8	52	90	0.0
54	214.0	73.9	116	422	0.8

* In %, based on 2 aliquots of quality control plasma from same pool

CONCLUSIONS:

Plans for the next year are as follows:

1. Continue editing and telephone clarification
2. Data entry of non-scannable portions of the questionnaire
3. Scanning of the questionnaire
4. Preliminary tabulations of responses
5. Preparation and mailing of the second newsletter
6. Development of the second questionnaire
7. Complete 6½ year storage effects analyses and report results.

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Stability of Ascorbic Acid, Carotenoids, Retinol, and Tocopherols in Plasma Stored at −70°C for 4 Years[1]

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Abstract

Aliquots from 40 plasma pools preserved with metaphosphoric acid were assayed for their ascorbic acid values after 12, 24, and 42 months of storage at −70°C. Similarly, aliquots from 16 plasma pools were assayed for values of retinol, several carotenoids, and two tocopherols at 15.5, 27.5, and 51.5 months of storage at −70°C. There were no indications of important losses of these antioxidant micronutrients during storage from the first to the last assay.

Introduction

The value of serum banks for studies of acute infectious diseases has long been recognized (1). Their use for studies of chronic diseases is more recent and is expanding rapidly (2). In view of this widespread use, there is need for elementary knowledge that is currently lacking. An example is the effect of long-term freezer storage on serum or plasma values of many analytes. What little is known about storage stability of antioxidant micronutrients is essentially limited to retinol, β-carotene, and α-tocopherol; it is almost entirely based on observational studies in which the source population is different for each storage time (3). In such studies, changes with duration of storage can be confounded by any differences in serum or plasma concentrations in the various populations.

Much better estimates can be obtained by assays of aliquots of the same serum or plasma pools stored and assayed after different lengths of storage. However, only a few studies have reported on repeated assays of aliquots of the same specimens after storage times of more than 1 year. Ascorbic acid showed only "marginal degradation" after storage at −70°C for 1.5 years when preserved with DTT (4). Serum from 35 women that had been stabilized with metaphosphoric acid showed concentrations of ascorbic acid and dehydroascorbic acid after 4–6 years storage at −70°C that were "generally similar" to fresh drawn samples (5). Retinol was stable for periods up to 8 years both at −20 and −70°C (6–7). No detectable changes were noted in serum values of carotenoids and α-tocopherol after 15 months of storage at −70°C (8), whereas considerable losses of carotenoids were noted after storage at −20°C for as short a time as 6 months (9).

To add to these data, we have periodically assayed aliquots of several large plasma pools. This report records our results for ascorbic acid after 1.0, 2.0, and 3.5 years of storage, and α- and β-carotene, cryptoxanthin, lutein, lycopene, and α- and γ-tocopherol after 1.3, 2.3, and 4.3 years of storage at −70°C.

Materials and Methods

From June to November 1989, a mass campaign was conducted in Washington County, MD, to recruit volunteers to donate 20 ml of blood for a research bank. Blood was drawn into 20-ml heparinized vacutainers (Fisher Scientific, Pittsburgh, PA). The tubes were tilted slowly back and forth to 45° from the horizontal for a total of 8 times to ensure that the heparin dissolved. Blood was refrigerated at 4°C until it was processed, usually within 2–6 h, and always within 24 h of blood drawing. The vacutainers were centrifuged at 1000 × *g* for 30 min. For the ascorbic acid assays, 0.7 ml of plasma was pipetted into 5-ml blue-capped Vanguard Cryogenic Vials (Sumitomo Bakelite Co., Ltd., Neptune, NJ) containing 0.7 ml of 10% solution of metaphosphoric acid. Equal aliquots of the remaining plasma were pipetted into each of two 5-ml Cryotubes with clear caps to distinguish them from the aliquots for ascorbic acid assays. All aliquots were quickly frozen at −70°C and kept at approximately that temperature until they were withdrawn for various studies.

Plasma for the study of storage effects came from persons who resided in states not contiguous to Washington County. They were selected from specimens donated closest to the end of the blood donation campaign, mostly in October and November 1989. Each pool was composed of plasma from four persons. For ascorbic acid, there were a total of 44 pools, 10 from each of 4 age-sex groups (males born 1910–1939; females born 1910–1939; males born 1940–1969, and females born 1940–1969) plus 4 "comparison" pools derived from a single large pool to estimate intraassay variation at each storage time. For other analytes, there were only 16 pools, 4 from each of the 4 age-sex groups plus 2 additional comparison pools.

The specimens designated for each pool were removed from the freezers and thawed in ice water under dim yellow light. They were then inverted 20 times to remove any possible effects of layering during freezing. After each pool was prepared, 6 aliquots were pipetted into Cryotubes, labeled with pool numbers, and promptly refrozen having been thawed for <30 min. At the designated storage times, one of these aliquots from each pool was shipped in insulated containers containing dry ice to the assay laboratory, where the specimens were stored at −85°C until thawed for assay.

Received 10/20/94; revised 3/23/95; accepted 3/24/95.

[1] Support for this study was provided by National Cancer Institute Research Grant CA 47503 and Preventive Oncology Academic Award CA 01522, and National Heart, Lung, and Blood Institute Research Career Award HL 21670.

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Assays for ascorbic acid were done at 12, 24, and 42 months after the midpoint of the blood donation dates for the contributors to these pools. For retinol, carotenoids, and tocopherols, assays were done at 15.5, 27.5, and 51.5 months, respectively, after their midpoint collection dates. The laboratory was masked with regard to the source of each aliquot.

For ascorbic acid assays, metaphosphoric acid-stabilized plasma samples were thawed at room temperature while being protected from light (20 min), mixed by vortex (15 s), and then centrifuged (15 min; 4°C; 10,000 × *g*). The clear supernatant was assayed for ascorbic acid with the use of 2,4-dinitrophenylhydrazine as chromogen (10, 11). The laboratory accuracy for this analytical procedure with the use of both internally and externally prepared quality control specimens is ± 4%, whereas the day-to-day and within-day precision (coefficient of variation) of the laboratory for the assay is <0.05. The analytical instrumentation used to assay ascorbic acid did not vary throughout the 42 months of assay. This equipment consisted of a Gilford Response UV/Vis spectrophotometer (CIBA-Corning Diagnostics Corp., Medfield, MA) plus a Gilford printer/plotter. They were maintained continually under the manufacturer's authorized service agreements. UV/Vis lamps were replaced as necessary.

The other frozen plasma samples were thawed at room temperature while being protected from light (20 min), mixed by vortex (15 s), and then centrifuged (2500 × *g*; 5 min; 4°C) to obtain clear samples that were assayed for the fat-soluble vitamins and carotenoids with the use of reversed-phase HPLC methodology (8). Laboratory accuracy for this analytical procedure, based on internally and externally prepared quality controls, is 2% for retinol, <5% for tocopherols, and <12% for the individual carotenoids, whereas the day-to-day and within-day precision (coefficient of variation) of the laboratory for these fat-soluble analytes is <0.06. The analytical instrumentation used to assay the fat soluble vitamins and carotenoids did not vary throughout the 51.5 months of assay. This equipment included a ternary-gradient HPLC pump (Model SP8800; Thermo Separation Products, Fremont, CA); a dynamic mixer (Model SP5800; Thermo Separation Products); an 80 position autosampler (Model SP8775; Thermo Separation Products) equipped with a 20-μl sample loop; a guard column (NewGuard C_{18}; Applied Biosystems, Inc., Foster City, CA); an analytical HPLC column (3 μm and 4.6 x 100 mm; Microsorb C_{18}, Rainin Instrument Co., Inc., Woburn, MA); and two analytical, variable wavelength, programmable UV/VIS detectors (Model 783, Kratos Analytical; Applied Biosystems, Inc.) interfaced with two dual-channel, programmable integrators (Model SP4270; Thermo Separation Products). All instrumentation was maintained continually under the manufacturer's authorized service agreement. UV and Vis lamps were replaced as necessary as were the guard and analytical HPLC columns.

To estimate the average rate of change for each of the selected micronutrients over the entire observation period and the baseline value for a storage time of zero, a simple linear regression model was fitted for the concentration of each pool at the three storage times (measured in years) with the use of the least squares method. The intercept represents the estimated "baseline" value. The slope divided by the intercept and multiplied by 100 represents the percentage change in concentration per year, expressed as a percentage of the baseline value.

Results

Intraassay coefficients of variation were based on the comparison specimens that had been assayed along with other specimens at each storage time. Calculations were based on values for the four comparison aliquots in each ascorbic acid set and the two comparison aliquots in sets assayed for retinol, carotenoids, and tocopherols. Table 1 shows that intraassay variability was the least for retinol and ascorbic acid and the greatest for α- and β-carotenes.

Table 1 Intraassay coefficients of variation[a] for concentrations of selected plasma vitamins at each of the three assay times

Vitamins	Assays		
	First	Second	Third
Ascorbic acid	1.7	1.4	3.5
Retinol	1.5	2.6	1.8
Carotenoids			
α-Carotene	10.6	12.2	12.6
β-Carotene	12.9	14.3	16.8
Cryptoxanthin	7.3	9.5	6.1
Lutein	3.3	1.9	2.6
Lycopene	4.0	3.9	1.7
Tocopherols			
α-Tocopherol	8.4	7.1	7.8
γ-Tocopherol	5.8	9.2	10.6

[a] Expressed as percentages.

Table 2 Estimated baseline plasma concentrations of selected vitamins by age-sex groups

Vitamins	Units	Estimated baseline values			
		Males		Females	
		20–49 yrs	50–69 yrs	20–49 yrs	50–69 yrs
Ascorbic acid	mg/dl	1.08	1.15	1.23	1.58
Retinol	μg/dl	80.1	81.2	60.5	74.8
Carotenoids					
α-Carotene	μg/dl	3.3	4.9	5.5	6.3
β-Carotene	μg/dl	18.0	22.6	29.1	45.3
Cryptoxanthin	μg/dl	9.14	10.6	10.2	18.3
Lutein	μg/dl	22.6	29.8	23.6	37.4
Lycopene	μg/dl	79.1	50.9	48.9	78.1
Tocopherols					
α-Tocopherol	mg/dl	1.22	1.24	1.02	1.46
γ-Tocopherol	mg/dl	0.22	0.25	0.21	0.29

Calculated baseline values of the selected vitamins in plasma are shown in Table 2. Although the donors of plasma for the pools in this study were not selected to be representative of any particular population, it is reassuring that these values are compatible with other published baseline values from American populations (4, 12).

Table 3 shows the mean values at each storage time and the estimated average annual percentage changes in analytes during the period between first and third assays. All changes were slight and well within the limits of chance variability, with overall decreases for ascorbic acid, retinol, and tocopherols and increases for the carotenoids.

Discussion

Although questions can be raised about the fact that specimens in this study were frozen and then thawed some time later for

Table 3 Mean concentrations of selected vitamins in plasma at each of the three assay times, and estimated average percentage changes in concentration per year of storage at −70°C during the study period

Vitamins	Assays			Change per year (%)	
	First	Second	Third	Mean	SE
Ascorbic acid (mg/dl)	1.25	1.23	1.22	−0.66	0.32
Retinol (μg/dl)	73.56	73.68	72.68	−0.49	0.11
Carotenoids (mg/dl)					
α-Carotene	5.01	5.17	5.19	+0.75	0.45
β-Carotene	28.82	29.08	29.12	+0.32	0.30
Cryptoxanthin	12.09	12.42	12.44	+1.03	0.36
Lutein	28.41	28.86	28.68	+0.18	0.36
Lycopene	64.17	64.49	64.31	+0.03	0.19
Tocopherols (mg/dl)					
α-Tocopherol	1.23	1.22	1.21	−0.41	0.13
γ-Tocopherol	0.24	0.24	0.23	−0.92	0.24

the initial assays, this does not appear to be a problem. A number of studies have shown that even repeated freeze-thaw cycles have no demonstrable effect on concentrations of ascorbic acid in stabilized plasma or on concentrations of retinol, total carotenoids, β-carotene, lycopene, total tocopherols, or α-tocopherol.

It is also possible that losses of analytes are greater during the early period of storage than they are later. This study could not detect early losses because the first assays were not done until approximately 1 year after storage was begun. Again, the results of prior studies do not support such a possibility (6, 7, 13–17). Almost all of them involved short periods of storage; at −70°C, losses were inconsequential. If there are only slight losses during the early storage periods, extrapolation from the later assays in the present study, also with slight losses, should give estimates of the true baseline values with relatively little error.

Although the present study encompasses a range of only 3.5–4.3 years, it is the most comprehensive report of the effects of long-term freezer storage of several important antioxidant micronutrients. The effects on ascorbic acid are particularly noteworthy because of the limited information on its changes in concentration when acidified plasma is stored (5). The fact that plasma values of ascorbic acid, retinol, carotenoids, and tocopherols remained almost constant for several years should be encouraging to all investigators of serological precursors of cancer and other diseases.

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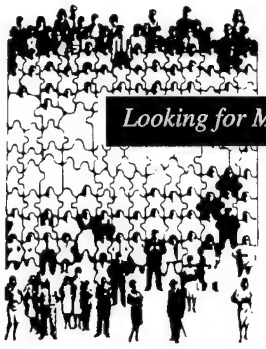

JOHNS HOPKINS RESEARCH CENTER
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Study Number_____

INTRODUCTION

Please follow the instructions carefully and answer all the questions unless otherwise instructed. Then return the completed questionnaire in the stamped envelope provided. All of the information you provide is confidential.

Thank you very much for your valuable help with this research.

INSTRUCTIONS

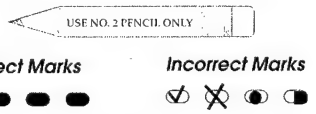
1. **Read each question carefully. Then use a no. 2 pencil to answer by filling in the blank space and darkening the circles.**

   Example: What is your date of birth?
   (Write in date as shown)

MONTH		DAY		YEAR	
0	1	0	8	3	0
●	○0	●	○0	○0	●
○1	●	○1	○1	○1	○1
	○2	○2	○2	○2	○2
	○3	○3	○3	●	○3
	○4		○4	○4	○4
	○5		○5	○5	○5
	○6		○6	○6	○6
	○7		○7	○7	○7
	○8		●	○8	○8
	○9		○9	○9	○9

MARKING INSTRUCTIONS

Use a No. 2 pencil only.
Make heavy black marks that darken the circle completely.
If you change your mind, please erase completely.



2. **Unless the instructions tell you otherwise, darken only one circle.**

3. **Some questions have instructions next to the answer telling you to skip questions which do not apply to you. First darken the circle. Then follow the skip as directed.**

PLEASE CHECK THE INFORMATION BELOW AND CORRECT THE INFORMATION IF THERE IS A MISTAKE.

DO NOT MARK IN THIS AREA

THANK YOU!

If you have any questions, please feel free to call our office at (301) 791-3230

CURRENT HEALTH STATUS

1. What is your date of birth?
(Write in number and darken circles)

MONTH		DAY		YEAR	
0	0	0	0	0	0
1	1	1	1	1	1
	2	2	2	2	2
	3	3	3	3	3
	4		4	4	4
	5		5	5	5
	6		6	6	6
	7		7	7	7
	8		8	8	8
	9		9	9	9

2. How much do you weigh?
(Write in number and darken circles)

POUNDS		
0	0	0
1	1	1
2	2	2
3	3	3
4	4	4
5	5	5
	6	6
	7	7
	8	8
	9	9

3. What is your marital status?

- ○ Never married
- ○ Married
- ○ Widowed
- ○ Divorced
- ○ Separated

4. After you were 21 years old, about how many times have you GAINED AND LOST 10 or more pounds? (NOT counting PREGNANCY and NURSING.)

- ○ Never
- ○ 1-5 times
- ○ 6-10 times
- ○ 11-15 times
- ○ 16 or more times

HEALTH HISTORY

5. Have you ever been told by a doctor or other health professional that you have any of the conditions listed below?

	No	Yes	How old were you when you were first told you had this condition?
a. Diabetes	○	○	_____
b. High cholesterol	○	○	_____
c. Heart attack	○	○	_____
d. Angina pectoris	○	○	_____
e. Stroke	○	○	_____
f. TIA (transient ischemic attack)	○	○	_____
g. Peripheral artery disease or claudication of legs (pain with walking or exercise) (not varicose veins)	○	○	_____
h. Osteoporosis	○	○	_____
i. Hip fractures	○	○	_____
j. Wrist or Colles' fracture	○	○	_____
k. Fibrocystic disease of the breast or other benign breast disease	○	○	_____
l. Endometriosis	○	○	_____
m. Uterine fibroids	○	○	_____
n. High blood pressure (excluding during pregnancy)	○	○	_____
o. Migraine headaches	○	○	_____
p. Thyroid disease	○	○	_____
q. Rheumatoid arthritis	○	○	_____
r. Gallbladder disease	○	○	_____
s. Gastric or duodenal ulcer	○	○	_____
t. Macular degeneration of the retina	○	○	_____

Question 5 continued from page 2

		No	Yes	How old were you when you were first told you had this condition?
u.	Cataract	○	○	_____
v.	Asthma	○	○	_____
w.	Emphysema or chronic bronchitis	○	○	_____
x.	Diverticulitis/diverticulosis	○	○	_____
y.	Parkinson's disease	○	○	_____
z.	Kidney stones	○	○	_____
aa.	Ulcerative colitis/Crohn's disease	○	○	_____
bb.	Breast cancer	○	○	_____
cc.	Cancer of the cervix (Include in-situ)	○	○	_____
dd.	Cancer of the uterus (endometrium)	○	○	_____
ee.	Cancer of the ovary	○	○	_____
ff.	Colon or rectal polyp (benign)	○	○	_____
gg.	Cancer of the colon or rectum	○	○	_____
hh.	Cancer of the lung	○	○	_____
ii.	Melanoma	○	○	_____
jj.	Basal cell skin cancer	○	○	_____
kk.	Squamous cell skin cancer	○	○	_____
ll.	Prostate cancer	○	○	_____
mm.	Other cancer (Specify site of other cancer)	○	○	_____
nn.	Other major illness (specify illness)	○	○	_____ _____ _____

6. Have you ever had any of the following surgical operations?

Operation or Surgery	How old were you when you had this surgery?	Where was this surgery done?
BREAST BIOPSY OR LUMPECTOMY (removal of breast tissue) ○ No ○ Yes If yes, number of biopsies or lumpectomies ○ One ○ Two ○ Three or more How old were you when you had your MOST RECENT BIOPSY? Did the biopsies show breast cancer? ○ No ○ Yes	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
MASTECTOMY (removal of a breast) ○ No ○ Yes If yes, how many breasts were removed? ○ One breast ○ Both breasts	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____

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DO NOT MARK IN THIS AREA

6. Have you ever had any of the following surgical operations? (Continued)

Operation or Surgery	How old were you when you had this surgery?	Where was this surgery done?
HYSTERECTOMY (removal of uterus) ○ No ○ Yes	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
OOPHORECTOMY (removal of ovaries) ○ No ○ Yes ○ One ovary ○ Both ovaries	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
PROSTATE SURGERY ○ No ○ Yes If yes, what type of surgery? Transurethral Resection (TURP) ○ No ○ Yes Biopsy ○ No ○ Yes Prostatectomy (removal of the prostate gland) ○ No ○ Yes	_____ Years old _____ Years old _____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
VASECTOMY (male sterilization) ○ No ○ Yes	_____ Years old	○ Washington County Hospital ○ Other, specify hospital, city, state: _____ _____
OTHER SURGERIES ○ No ○ Yes If yes, what other surgeries have you had? _____ _____ _____	_____ Years old _____ Years old _____ Years old	○ Washington County Hospital ○ Other _____ ○ Washington County Hospital ○ Other _____ ○ Washington County Hospital ○ Other _____

FAMILY HISTORY

7. Have any of the following blood-related relatives ever had cancer?(Do not count foster or step parents)

Relative	Ever had cancer			Type(s) of cancer	Age when cancer was found
Mother	○ No	○ Yes	○ Don't know	_____	_____ Years old
Mother's Mother	○ No	○ Yes	○ Don't know	_____	_____ Years old
Mother's Father	○ No	○ Yes	○ Don't know	_____	_____ Years old
Father	○ No	○ Yes	○ Don't know	_____	_____ Years old
Father's Mother	○ No	○ Yes	○ Don't know	_____	_____ Years old
Father's Father	○ No	○ Yes	○ Don't know	_____	_____ Years old

8. How many blood related SISTERS do you have?

○ None (Go to Question 11) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five

9. How many of your blood related SISTERS ever had cancer?

○ None (Go to Question 11) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five ○ Don't know

10. For each of your blood related SISTERS who ever had cancer, please list the type(s) of cancer and how old your SISTER was when the cancer was found.

Sister #	Type(s) of cancer	Age when cancer was found
○ 1	_____	_____ years old
○ 2	_____	_____ years old
○ 3	_____	_____ years old

11. How many blood related BROTHERS do you have?

○ None (Go to Question 14) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five

12. How many of your blood related BROTHERS ever had cancer?

○ None (Go to Question 14) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five ○ Don't know

13. For each of your blood related BROTHERS who ever had cancer, please list the type(s) of cancer and how old your BROTHER was when the cancer was found.

Brother #	Type(s) of cancer	Age when cancer was found
○ 1	_____	_____ years old
○ 2	_____	_____ years old
○ 3	_____	_____ years old

14. How many blood related CHILDREN do you have? (Do not count, adopted, foster, or step children)

○ None (Go to Question 17) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five

15. How many of your blood related CHILDREN ever had cancer?

○ None (Go to Question 17) ○ Two ○ Four ○ Six or more
○ One ○ Three ○ Five ○ Don't know

16. For each of your blood related CHILDREN who ever had cancer, please list the type(s) of cancer and how old your CHILD was when the cancer was found.

Child #	Type(s) of cancer	Age when cancer was found
○ 1	_____	_____ years old
○ 2	_____	_____ years old
○ 3	_____	_____ years old

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DO NOT MARK IN THIS AREA

17. How often on average have you taken any of the following medications?

MEDICATION	NUMBER OF PILLS TAKEN: None	Less than 1 per week	1-3 per week	4-6 per week	1 per day	2 per day	3 per day	4 or more per day	DOSE / STRENGTH: Baby	Regular (325mg)	Extra Strength (500 mg)	Don't Know	DURATION — How many years taken?: 1	2-4	5-9	10-14	15-19	20-24	25+	Are you taking now? NO	YES
Acetaminophin (for example, Tylenol, Anacin-3 or other brands)	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Aspirin (for example, Bufferin, AlkaSeltzer, Bayer, Ecotrin or other brands)	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Other Anti-Inflammatory (for example Ibuprofen, Advil, Ibuprin, Motrin, Excedrin IB, Aleve, Ansaid, Naproxen, Naprosyn, Sulindac, Orudis, Piroxicam, Nalfon, or other brands) Usual Brand: ______ ______ ______	○	○	○	○	○	○	○	○					○	○	○	○	○	○	○	○	○
Melatonin	○	○	○	○	○	○	○	○					○	○	○	○	○	○	○	○	○

18. Have you ever taken any MULTIPLE vitamins or minerals regularly (at least once per week)?
○ No (Go to Question 19) ○ Yes (Please complete the following):

Brand Name	How many Years taken?: 1or less	2-4	5-9	10-14	15-19	20-24	25+	Usual Number of Tablets or Capsules taken: Less Than 1 per week	One per week	2-3 per week	4-6 per week	1 per day	More than 1 per day	Are you taking now? No	Yes
______	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
______	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
______	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
______	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
______	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
______	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
______	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
______	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

19. Have you ever taken any INDIVIDUAL vitamin or INDIVIDUAL mineral regularly (at least once per week)?

○ No (Go to Question 20) ○ Yes (Please complete the following):

Name of INDIVIDUAL Vitamin or Mineral	Dose per tablet or capsule	How many Years INDIVIDUAL VITAMIN OR MINERAL taken?							Usual Number of Tablets or Capsules taken						Are you taking now?	
		1or less	2-4	5-9	10-14	15-19	20-24	25+	Less Than 1 per week	One per week	2-3 per week	4-6 per week	1 per day	More than 1 per day	No	Yes
Example Vitamin C	400 mg.															
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
		○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

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20. Since 1989, have you had a colonoscopy or sigmoidoscopy (examination of the colon)?

○ NO ○ YES

22. What was the cause of infertility? Mark all that apply.

○ Did not look for cause
○ Tubal blockage
○ Ovulation / hormonal problem
○ Endometriosis
○ Cervical mucus factor
○ Cause not found
○ Male infertility
○ Other, specify ____________

24. What treatments did you have for infertility? Mark all that apply.

○ Surgery
○ Pergonal
○ Chlomid
○ Other, specify ____________

21. Have you and your spouse (or partner) ever tried to become <u>PREGNANT</u> for more than one year without success?

○ NO (Go to Question 26) ○ YES ○ NOT APPLICABLE (Go to Question 26)

23. Were you treated for infertility?

○ NO (Go to Question 26) ○ YES

25. Were the treatments successful?

○ NO ○ YES ○ DON'T KNOW

DO NOT MARK IN THIS AREA

26. **Have you ever used any of the following?**

- ○ Cigars
- ○ Pipes
- ○ Snuff
- ○ Chewing tobacco
- ○ Cigarettes
- ○ None

If you've never smoked cigarettes, go to question 31.

27. **At what ages were you smoking? (Complete all that apply)**

- ○ 5 - 14 years old
- ○ 15 - 24 years old
- ○ 25 - 34 years old
- ○ 35 - 44 years old
- ○ 45 - 54 years old
- ○ 55 - 64 years old
- ○ 65 years or older

Total number of years smoked cigarettes_____

28. **How many cigarettes do you or did you usually smoke each day?**

- ○ Less than 1 per day
- ○ 1-4
- ○ 5-14
- ○ 15-24
- ○ 25-34
- ○ 35 or more

29. **Have you ever stopped smoking for 6 months or more?**

- ○ NO (Go to Question 31)
- ○ YES

30. **If YES, how many times have you stopped smoking for 6 months or more?**

- ○ Once
- ○ Twice
- ○ Three times
- ○ Four times or more

31. **Before you were 21 years old, how many years did you work in the same room or live with someone who smoked cigarettes?**

Number of years **(Write in number and darken circles)**

(0)	0
(1)	1
(2)	2
(3)	3
(4)	4
(5)	5
(6)	6
	7
	8
	9

32. **After you were 21 years old, how many years did you work in the same room or live with someone who smoked cigarettes?**

Number of years **(Write in number and darken circles)**

(0)	0
(1)	1
(2)	2
(3)	3
(4)	4
(5)	5
(6)	6
(7)	7
(8)	8
(9)	9

33. **When you lived or worked with someone who smoked cigarettes, on average how many hours per day were you exposed to someone else's smoke?**

- ○ 0 Hours
- ○ 1 - 3 Hours
- ○ 4 - 6
- ○ 7 - 10
- ○ 11 - 16
- ○ More than 17 hours

34. Have you ever drunk alcoholic beverages (such as beer, wine or liquor) at least once a month?

○ No (Go to Question 40) ○ Yes

35. How old were you when you FIRST STARTED drinking alcoholic beverages at least once a month?

Years Old (Write in number and darken circles)

⓪	0
①	1
②	2
③	3
④	4
⑤	5
⑥	6
⑦	7
	8
	9

36. Do you drink alcoholic beverages at least once a month NOW?

○ No ○ Yes

37. Considering the time you may have stopped drinking and then restarted, how many TOTAL YEARS have you actually drunk alcohol?

Number of years (Write in number and darken circles)

⓪	0
①	1
②	2
③	3
④	4
⑤	5
⑥	6
⑦	7
⑧	8
⑨	9

38. How many drinks of alcoholic beverage do/did you USUALLY have PER WEEK? (Consider a drink to be a drink or shot of liquor, a 4oz. serving of wine, or one 12oz. can or bottle of beer, light beer, or a wine cooler.)

Number of drinks per week (Write in number and darken circles)

⓪	⓪
①	①
②	②
③	③
④	④
⑤	⑤
⑥	⑥
⑦	⑦
⑧	⑧
⑨	⑨

39. On how many DAYS do/did you drink each of the following?

	None	1-3 days per month	1 day per week	2-3 days per week	4-6 days per week	Every day
Beer	○	○	○	○	○	○
Wine	○	○	○	○	○	○
Liquor	○	○	○	○	○	○

40. The questions in this section ask about whether, and how often, you ate certain kinds of meat during the LAST 12 MONTHS.

For each of the foods you eat, please mark how often you eat it and how it was cooked.

During the LAST 12 MONTHS or so, how often did you eat...	AVERAGE USE LAST 12 MONTHS									What was the one most common method of cooking?(check one)	How was it usually cooked on the outside?
	Never or seldom	1 per month	2-3 per month	1 per week	2 per week	3-4 per week	5-6 per week	1 per day	2+ per day		
A. Hamburger/ Cheeseburger?	0	0	0	0	0	0	0	0	0	0 Pan fried 0 Oven-broiled 0 Grilled/barbecued 0 Other	0 Not browned or 0 Slightly browned 0 Medium brown 0 Well browned 0 Blackened/charred
B. Beef Steak?	0	0	0	0	0	0	0	0	0	0 Pan fried 0 Oven-broiled 0 Grilled/barbecued 0 Other	0 Not browned or 0 Slightly browned 0 Medium brown 0 Well browned 0 Blackened/charred
C. Fried Chicken?	0	0	0	0	0	0	0	0	0	0 Pan fried 0 Oven-Fried 0 Deep Fried	0 Not browned or 0 Slightly browned 0 Medium brown 0 Well browned 0 Blackened/charred
D. Chicken (other than fried)?	0	0	0	0	0	0	0	0	0	0 Oven-Baked 0 Grilled/barbecued 0 Broiled 0 Other	0 Not browned or 0 Slightly browned 0 Medium brown 0 Well browned 0 Blackened/charred
E. Pork Chops?	0	0	0	0	0	0	0	0	0	0 Pan fried 0 Oven-broiled 0 Grilled/barbecued 0 Other	0 Not browned or 0 Slightly browned 0 Medium brown 0 Well browned 0 Blackened/charred
F. Bacon?	0	0	0	0	0	0	0	0	0	0 Pan fried 0 Oven-broiled 0 Grilled/barbecued 0 Other	0 Not browned or 0 Slightly browned 0 Medium brown 0 Well browned 0 Blackened/charred
G. Fish?	0	0	0	0	0	0	0	0	0	0 Pan fried 0 Oven-broiled 0 Grilled/barbecued 0 Other	0 Not browned or 0 Slightly browned 0 Medium brown 0 Well browned 0 Blackened/charred

DO NOT MARK IN THIS AREA

41. When you were an infant were you breast fed or bottle fed?

() Breast fed () Bottle fed () Both breast and bottle fed () Don't Know

42. When did your hair begin to gray? By graying we mean the appearance of more than just a few gray hairs.

() Not yet
() Less than 20 years old
() 20 to 24 years old
() 25 to 29 years old
() 30 to 34 years old
() 35 to 39 years old
() 40 to 44 years old
() 45 to 49 years old
() 50 years or older

43. Have you ever used hair coloring products for more than 6 months?

() No (Men: Continue with question 47)
(Women: Continue with Question 53)
() Yes (Please complete the following)

First answer "Yes" or "No" for each type of hair color product listed. If you answered "Yes" in Column A then answer the questions in Columns B, C, D & E for that product. If answered "No" then go to the next hair coloring product.

A Have you ever used this product?	B At What Age Did You Start Using This Product?	C How Many Years Have You Used This?	D How Often Did You Apply This Coloring Product?	E What Color Did You Usually Use?
44. Temporary Rinses (Color removed by 1-2 shampoos) () Yes → () No (Go to question 45)	_____Years Old	_____Years	() 1-2 times per year () 3-10 times per year () 11-20 times per year () 21-40 times per year () 40 or more times per year	() Brown () Black () Red () Blond () Silver toners () Other
45. Semi-permanent products (Color washed out by 6-12 shampoos) () Yes → () No (Go to question 46)	_____Years Old	_____Years	() 1-2 times per year () 3-10 times per year () 11-20 times per year () 21-40 times per year () 40 or more times per year	() Brown () Black () Red () Blond () Silver toners () Other
46. Permanent products (Color does not wash out when you shampoo your hair) () Yes → () No Men: (Go to question 47) Women: (Go to question 53)	_____Years Old	_____Years	() 1-2 times per year () 3-10 times per year () 11-20 times per year () 21-40 times per year () 40 or more times per year	() Brown () Black () Red () Blond () Silver toners () Other

MEN: PLEASE COMPLETE QUESTIONS 47 THROUGH 52
WOMEN: PLEASE GO TO QUESTION 53

47. A digital rectal exam is when a doctor inserts his finger in the rectum to check for problems such as an enlarged prostate gland or polyps.

Have you ever had a digital rectal exam?

- ○ No (Go to Question 49)
- ○ Yes (Continue with Question 48)

48. How many years has it been since your last digital rectal exam?

- ○ Less than one year
- ○ one year
- ○ two years
- ○ three or more years

49. Have you ever had a blood test (PSA) to see if you had prostate cancer?

- ○ No (Go to Question 52)
- ○ Yes (Continue with Question 50)

50. Has your PSA blood test ever been abnormal?

- ○ No (Go to Question 52)
- ○ Yes (Continue with Question 51)

51. Was it followed up by: (Mark all that apply.)

- ○ Not followed up
- ○ Ultrasound
- ○ Biopsy
- ○ Surgical operation
- ○ Radiation
- ○ Hormones

52. Have you ever taken the following hormones?

			How many years did you take the hormones?						
			1 or less	2 - 4	5 - 9	10 - 14	15 - 19	20 - 24	25 +
Thyroid Hormones	○ No	○ Yes	○	○	○	○	○	○	○
Testosterone	○ No	○ Yes	○	○	○	○	○	○	○
Anabolic Steroids	○ No	○ Yes	○	○	○	○	○	○	○

MEN: PLEASE GO TO QUESTION 77
WOMEN: PLEASE COMPLETE THE FOLLOWING QUESTIONS

53. How old were you when you first started having MENSTRUAL PERIODS?

Age Periods Started **(Write in number and darken circles)**

⓪	⓪
①	①
②	②
	③
	④
	⑤
	⑥
	⑦
	⑧
	⑨

DO NOT MARK IN THIS AREA

54. Have your MENSTRUAL PERIODS stopped permanently?

- ○ Yes, menstrual periods stopped. (Continue with Question 55)
- ○ Had menopause, but now have periods due to hormone replacement therapy. (Continue with Question 55)
- ○ No, still menstruating (Go to Question 57)
- ○ Not sure (Go to Question 57)

55. How old were you when your natural MENSTRUAL PERIODS stopped?

Age Periods Stopped (Write in number and darken circles)

⓪	⓪
①	①
②	②
③	③
④	④
⑤	⑤
	⑥
	⑦
	⑧
	⑨

56. Why did your natural MENSTRUAL PERIODS stop?

- ○ Surgical Menopause (Hysterectomy or removal of uterus)
- ○ Natural menopause (Change of Life)
- ○ Other, specify_____

57. Have you ever taken BIRTH CONTROL PILLS (oral contraceptives)?

- ○ No (Go to Question 60)
- ○ Yes (Continue with Question 58)

58. At what age did you first use birth control pills?

⓪	⓪
①	①
②	②
③	③
④	④
	⑤
	⑥
	⑦
	⑧
	⑨

(Write in number and darken circles)

59. Altogether, how many years did you take BIRTH CONTROL PILLS (oral contraceptives)?

Total Number of Years

⓪	⓪
①	①
②	②
③	③
	④
	⑤
	⑥
	⑦
	⑧
	⑨

(Write in number and darken circles)

60. Have you EVER taken ESTROGENS (hormones, such as Premarin) alone or in combination with progestin for symptoms or effects of menopause?

- ○ No (Go to Question 65)
- ○ Yes (Continue with Question 61)

61. At what age did you first use estrogens for effects of menopause?

⓪	⓪
①	①
②	②
③	③
④	④
⑤	⑤
⑥	⑥
⑦	⑦
⑧	⑧
⑨	⑨

(Write in number and darken circles)

62. Are you currently taking ESTROGENS?

- ○ No
- ○ Yes

63. Altogether, how many years did you take ESTROGENS?

Total number of years (Write in number and darken circles)

⓪	⓪
①	①
②	②
③	③
④	④
	⑤
	⑥
	⑦
	⑧
	⑨

64. What type of estrogens do/did you use?

- ○ Patch
- ○ Pills
- ○ Shots
- ○ Vaginal creams or suppositories
- ○ Not sure
- ○ Other, specify:_____

65. Have you ever taken PROGESTINS (hormones such as Provera) alone or in combination with estrogens for symptoms or effects of MENOPAUSE?

- ○ No (Go to Question 69)
- ○ Yes

DO NOT MARK IN THIS AREA

66. At what age did you first use PROGESTINS?

0	0
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9

(Write in number and darken circles)

67. Are you currently taking PROGESTINS?

- ○ **No**
- ○ **Yes**

68. Altogether, how many years did you take PROGESTINS?

Total Number of Years

0	0
1	1
2	2
3	3
4	4
	5
	6
	7
	8
	9

(Write in number and darken circles)

69. Have you ever taken thyroid hormones?

- ○ **No (Go to Question 71)**
- ○ **Yes (Continue with Question 70)**

70. How many years did you take thyroid hormones?

0 - 1	2 - 4	5 - 9	10 - 14	15 - 19	20 - 24	25+
○	○	○	○	○	○	○

71. Have you ever been pregnant?

- ○ **No (Go to Question 77)**
- ○ **Yes (Continue with Question 72)**

72. For each time you became pregnant, please mark the outcome of the PREGNANCY.

	PREGNANCY OUTCOME			
	Live Birth	Stillborn	Miscarriage	Abortion
1st Pregnancy	○	○	○	○
2nd Pregnancy	○	○	○	○
3rd Pregnancy	○	○	○	○
4th Pregnancy	○	○	○	○
5th Pregnancy	○	○	○	○
6th Pregnancy	○	○	○	○
7th Pregnancy	○	○	○	○
8th Pregnancy	○	○	○	○
9th Pregnancy	○	○	○	○
10th Pregnancy	○	○	○	○
11th Pregnancy	○	○	○	○

73. How old were you when your first child was born?

Age

0	0
1	1
2	2
3	3
4	4
5	5
	6
	7
	8
	9

(Write in number and darken circles)

○ Does not apply

74. Are you pregnant now?

○ No

○ Yes

75. Did you breastfeed any of your children?

○ No (Go to Question 77)

○ Yes (Continue with Question 76)

76. In total, how many months of your life have you spent breast feeding?

Months

0	0
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9

(Write in number and darken circles)

77. PLEASE INDICATE THE NAME OF SOMEONE AT A DIFFERENT ADDRESS THAT WE MIGHT WRITE TO IN THE EVENT WE ARE UNABLE TO CONTACT YOU.

Name: _____ Relationship: _____
First Middle Last

Address: _____

_____

DO NOT MARK IN THIS AREA

THANK YOU FOR COMPLETING THE QUESTIONNAIRE!

Funding period: 10/1/94 - 9/30/98

TIME SCHEDULE

ACTIVE FOLLOW-UP OF CLUE II

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YEAR 01

10/1/94 - 9/30/95	• Updating of participant addresses • Preliminary preparation of questionnaire

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YEAR 02

10/1/95 - 2/28/96	• Establishment of computer data bases • Continue updating participant addresses • Preparation of questionnaire • Preparation of newsletter • Mail newsletter to pilot group
3/96	• Final mock-up and printing of questionnaire for pilot completed • Mailing of pilot questionnaire
4/96	• Data entry of pilot questionnaire • Mailing of newsletter • Address correction from mailing
5/96	• Alterations of questionnaire based on pilot
6/96	• Final questionnaire approval for printing
7/96	• Mailing of questionnaire
8/96	• Begin data entry
9/96	• Continue data entry/scanning • Telephone follow-up to clarify responses • Reminder postcards sent • Second mailing of questionnaires

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YEAR 03

10/1/96 - 9/30/97	• Continue data entry/scanning • Continue to locate addresses
2/97	• Preparation of second newsletter
4/97	• Mailing of second newsletter • Updating of addresses • Preparation of second round questionnaire

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YEAR 04

10/1/97 - 9/30/98	• Mailing of second round questionnaire • Begin data entry for second round • Preparation of food frequency questionnaire
4/98	• Mailing of third newsletter

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YEAR 05

10/1/98 - 9/30/99	• Mailing of food frequency questionnaire • Data entry • Data linkage and clean-up

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